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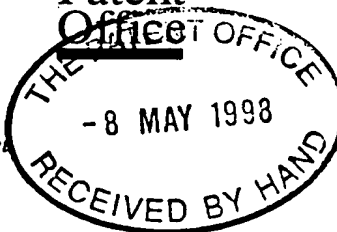
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2. Patent application number *(The Patent Office will fill in this part)*	08 MAY 1998	**9809951.8**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

CAMBRIDGE UNIVERSITY TECHNICAL SERVICES LIMITED
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CAMBRIDGE
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GB

'695680900 3

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation

UNITED KINGDOM

4. Title of the invention

BINDING MOLECULES

5. Name of your agent *(if you have one)*

MEWBURN ELLIS

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)*

109006

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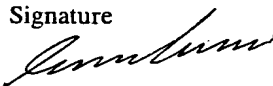|	
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11.   I/We request the grant of a patent on the basis of this application.

Signature   Date
                                                   7 May 1998

12. Name and daytime telephone number of person   SIMON M. KREMER   0117 926 6411
     to contact in the United Kingdom

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BINDING MOLECULES

TECHNICAL FIELD

5

The present invention relates to binding polypeptides having amino acid sequences derived from a modified constant region of the immunoglobulin G (IgG) heavy chain. The invention further relates to methods and
10   materials for producing such polypeptides, and methods and materials employing them.

PRIOR ART

15   *Immunoglobulins*

Immunoglobulins are glycoproteins which help to defend the host against infection. They generally consist of heavy and light chains, the N-terminal domains of which
20   form a variable or V domain capable of binding antigen. The V domain is associated with a constant or C-terminal domain which defines the class (and sometimes subclass [isotype], and allotype [isoallotype]) of the immunoglobulin.

25

Thus in mammalian species immunoglobulins exist as IgG, IgD, IgG, IgA and IgE; the IgG class in turn exists as 4 subclasses in humans (IgG1, IgG2, IgG3, IgG4). The C-domain in IgGs comprises three domains Cγ1, Cγ2, and Cγ3,
30   which are very similar between these subclasses (over 90% homology). The role of the subclasses appears to vary between species.

It is known that the C-domain is responsible for various
35   effector functions of the immunoglobulin (see Clark (1997) "IgG Effector Mechanisms" in "Antibody Engineering" Ed. Capra, Pub. Chem Immunol,

Basel,Kurger, Vol 65 pp 88-110, for a detailed review).

Briefly, IgG functions are generally achieved via interaction between the Fc region of the Ig and an Fcγ receptor (FcγR) receptor or other binding molecule, sometimes on an effector cell. This can trigger the effector cells to kill target cells to which the antibodies are bound through their variable (V) regions. Also antibodies directed against soluble antigens might form immune complexes which are targeted to FcγRs which result in the uptake (opsonisation) of the immune complexes or in the triggering of the effector cells and the release of cytokines.

In humans, three classes of FcγR have been characterised, although the situation is further complicated by the occurrence of multiple receptor forms. The three classes are:

(i) FcγRI (CD64) binds monomeric IgG with high affinity and is expressed on macrophages, monocytes, and sometimes neutrophils and eosinophils.

(ii) FcγRII (CD32) binds complexed IgG with medium to low affinity and is widely expressed. These receptors can be divided into two important types, FcγRIIa and FcγRIIb. The a form of the receptor is found on many cells involved in killing (eg macrophages, monocytes, neutrophils) and seems able to activate the killing process, and occurs as two alternative alleles. The b form seems to play a role in inhibitory processes and is found on B-cells and on mast cells and eosinophils. On B-cells it seems to function to suppress further immunoglobulin production and isotype switching to say for example the IgE class. On eosinophils and mast cells the b form may help to suppress activation of these cells through IgE binding to its separate receptor.

(iii) FcγRIII (CD16) binds IgG with medium to low affinity and is expressed on macrophages certain lymphocytes and killer cells, plus sometimes neutrophils, eosinophils and monocytes.

As well as binding to FcγRs, IgG antibodies can activate complement and this can also result in cell lysis, opsonisation or in cytokine release and inflammation. The Fc region also mediates such properties as the transportation of IgGs to the neonate (via the so-called 'FcRn'); increased half-life (also believed to be effected via an FcRn-type receptor - see Ghetie and Ward (1997) Immunology Today 18, 592-598) and self-aggregation. The Fc-region is also responsible for the interaction with protein A and protein G (which interaction appears to be analagous to the binding of FcRn).

Engineering immunoglobulins

Many of the Fc-mediated properties discussed above may be desirable in naturally occurring or artificially constructed antibodies. However, there are circumstances where, in particular, the cell killing, or the cytokine release and resulting inflammation, is inappropriate and undesirable.

Equally, however, it may be desirable to retain certain Fc-mediated functions, for instance the long plasma half life.

It is known that human IgG4, for example, does not activate complement and human IgG2 does not bind to the high affinity FcγRI receptor and so it has been previously used in some situations (TNF receptor fusion protein was made with IgG4 Fc).

However no human subclass lacks all of the relevant Fc effector triggering functions or complement activation in all circumstances, possibly owing to the existence of the several forms of the FcγRs. Thus, for instance, IgG4 can
5 trigger antibody dependent cellular cytotoxicity (ADCC) in some people and IgG2 binds to one allelic form of the FcγRIIa receptor and also activates complement.

An alternative approach has been to mutate the Fc
10 sequence to destroy residues crucial for function. Certain target residues have been identified and published (see review by Clark 1997, *supra*). These include the N-linked carbohydrate attached to the conserved site in the C_H2 domain, certain residues in the
15 lower hinge region (eg the sequence LLGGP) and a proline residue at position 331 and a sequence E-x-K-x-K at positions 318-322. One recent example is disclosed by Cole et al (1997) Journal of Immunology 159, 3613-3621. In that disclosure residues 234, 235 and 237 were mutated
20 to Alanines (or in the case of 235, sometimes to Glu). However these are all unusual residues at these positions in human IgG, thus the presence of such inappropriate amino acids may make the Fc more immunogenic or antigenic and may also lead to the loss of certain desirable Fc
25 functions.

Again this strategy has been used for the construction of a therapeutic aglycosylated CD3 antibody (see Routledge et al, 1993 Eur J Immunol 23: 403-411; see also UK PA
30 9206422.9) and for an inhibitory CD18 antibody. However the disadvantage here is that the new recombinant constructs have unusual sequences and may be recognised and rejected by the immune system as foreign.

35 Other approaches to modifying immunoglobulins are disclosed in WO 92/16562 (Lynxvale Ltd) which discusses modifying the allotype of the humanised IgG1 antibody

CAMPATH1H which has binding affinity for antigen CD52.
The CD52 antigen is found on human lymphocytes and
monocytes and has been used as a therapeutic target for
treatment of T and B-cell lymphomas and leukeamias,
immunosuppresion of organ and bone-marrow transplant
recipients and also treatment of some autoimmune and
related disorders such as rheumatoid arthritis and
systemic vasculitis.

WO 95/05468 (Lynxvale Ltd) also disclosed the
modification of allotypic determinants in Igs (or
derivatives) having desired binding or other effector
functions.

It can be seen from the forgoing that the provision of
methods or materials which would facilitate the
engineering of Fc regions such as to reduce unwanted
effects, while retaining or enhancing desirable
properties, would provide a contribution to the art.

DISCLOSURE OF THE INVENTION

The present inventors have used novel combinations of
human IgG subclass sequences to generate chimaeric
polypeptides comprising non-natural, human-mimicing Fc
sequences which nevertheless do not activate complement
or trigger cytotoxic activities through FcγR. At the same
time certain desirable IgG properties have been retained.
For instance the polypeptides do not contain 'non-human'
amino acids, and are therefore likely to have reduced
immunogenicity. Further, they still bind Protein A,
which is consistent with being able to cross the human
placenta through interaction with FcRn (neonatal Fc
receptor).

The manner by which the sequences were developed, and
certain demonstrated properties, will be discussed in

more detail hereinafter. However, briefly, the inventors
formulated numerous constructs based on three different
IgG sequeces (1, 2 and 4). Although the relevant regions
of these antibodies share homolgy, they do not precisely

5   correspond in terms of length, thereby complicating the
process of generating derivative sequences which retain
activities from the natural sequences. The constructs
were compared with the parental control antibodies in the
context of model antigen systems RhD and CD52 (CAMPATH-

10   1H). Surprisingly, a number of sequences were developed
with the required combination of activities not found in
the parent molecules.

     Thus in a first aspect of the present invention there is

15   disclosed a polypeptide binding molecule comprising (i) a
binding domain capable of binding a target molecule, and
(ii) an effector domain having an amino acid sequence
substantially homologous to all or part of the constant
domain of a human immunoglobulin heavy chain;

20   characterised in that the binding molecule is capable of
binding the target molecule without triggering
significant complement dependent lysis, or cell mediated
destruction of the target, and whereby the effector
domain is capable of specifically binding FcRn.

25
     The specific binding of FcRn may be evidenced by the
capability to specifically bind protein A.

     Thus the binding molecules according to the present

30   invention have improved clinical properties (e.g. in the
context of 'blocking' antibodies). This is achieved by
the provision of an Fc-derived effector domain which has
a reduced affinity for $Fc\gamma RI$, $Fc\gamma RIIa$ and $Fc\gamma RIII$, but
which retains the ability to bind protein A (and hence

35   FcRn, hence permitting neonatal transport and high half
life). Thus the residues responsible for binding FcRn in
IgGs need not be modified with respect to a natural Fc

region in the molecules of the present invention.

Although IgG1/IgG2 and IgG1/IgG4 chimeras have been
prepared in the past (see e.g. Morgan et al (1995)
Immunology 86: 319-324, or Chappel et al (1991) Proc Natl
Acad Sci USA 88: 9036-9040, or Greenwood et al (1993) Eur
J Immunol 23: 1098-1104) none of these has been shown to
have the combination of properties possessed by the
binding molecules of the present invention.

The various functions of the binding molecule can be
assessed without burden by those skilled in the art, for
instance by using methods as disclosed below, or methods
analagous to these. For instance, the FcγR binding
properties may be assessed directly, or indirectly e.g.
through inability to trigger monocyte chemiluminescence.

Specifically, the inability to trigger significant
complement dependent lysis (which will generally be
through a reduced affinity for the C1q molecule) can be
measured by CR-51 release from target cells in the
presence of the complement components e.g. in the form of
serum (as described below) whereby the binding molecule
causes less than 5%, preferably less than 2% specific
target cell lysis.

Similarly, cell mediated destruction of the target may be
assessed by CR-51 release from target cells in the
presence of suitable cytotoxic cells e.g. blood
mononuclear effector cells (as decribed below) whereby
the binding molecule causes less than 5%, preferably less
than 2% target cell lysis.

As an alternative to direct measurement, functionality
may be inferred by the ability to inhibit these
attributes in functional immunoglobulins. For instance
by providing a protective effect against the complement

lysis of cells, or the killing of cells (e.g. by ADCC), or by inhibiting the response of monocytes to sensitised cells, or the binding of immunoglobulins to FcγR bearing cells.

In one, preferred, embodiment of this aspect of the invention the effector domain comprises an amino acid sequence substantially homologous to the C_H2 sequence from human IgG1, G2 or G4, said sequence comprising the following amino acids or deletions at the stated positions, numbered with respect to the EU numbering system (see Kabat et al "Sequences of proteins of immunological interest". Bethesda, US Department of Health and Humna Services, NIH, 1991):

Posn	Amino acid
233	P
234	V
235	A
236	(No residue) or G
327	G
330	S

Several mutant immunoglobulins based on IgG1, IgG2, or IgG4 having the stated features, have been prepared and have shown to have the required properties. Although some of the individual residue mutations have been prepared in binding molecules of the prior art, the specified combination is novel, as is the achieved functionality.

Preferred forms of the binding molecule will now be discussed in more detail:

The effector domain

The peptide comprises an effector domain having an amino

- 9 -

acid sequence substantially homologous to all or part of a human immunoglobulin constant region, preferably an IgG C-domain.

5   Numerous sequences for human C regions have been published; see e.g. Clark (1997) supra. Other sequences for human immunoglobulin heavy chains can be obtained from the SwissProt and PIR databases using Lasergene software (DNAStar Limited, London UK) under accession

10   numbers A93433, B90563, A90564, B91668, A91723 and A02146 for human Igγ-1 chain C region, A93906, A92809, A90752, A93132, A02148 for human Igγ-2 chain C region, A90933, A90249, A02150 for human Igγ-4 chain C region, A92249, A91662, A02171 for human Igα-2 chain C region and A23511

15   for human Igγ-3 chain C region.

Homology (or identity, or similarity) may be assessed by any convenient method. Homology may be at the encoding nucleotide sequence or encoded amino acid sequence level.

20   By "substantially homologous" is meant that the comprised amino acid sequence shares at least about 50%, or 60%, or 70%, or 80% homology, most preferably at least about 90%, 95%, 96%, 97%, 98% or 99% homology with the reference immunoglobulin.

25

Similarity or homology may be as defined and determined by the TBLASTN program, of Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art, or, and this may be preferred, the standard program

30   BestFit, which is part of the Wisconsin Package, Version 8, September 1994, (Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA, Wisconsin 53711). BestFit makes an optimal alignment of the best segment of similarity between two sequences. Optimal alignments are

35   found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman.

This assessment can be made without burden by a person of ordinary skill in the art, in conjunction with assessing the required combination of activities, in order to recognise a molecule of the present invention.

5

In addition to having the reduced affinity for for $Fc\gamma RI$, $Fc\gamma RIIa$ and $Fc\gamma RIII$, it may be desirable that an ability to bind the 'inhibitory' receptor $Fc\gamma RIIb$ is retained or possessed by the effector molecule. Results obtained by

10 the present inventors indicate that the binding molecules which they have developed do have this property. Hitherto it was not appreciated in the art that the binding of Fc regions to $Fc\gamma RIIa$ and $Fc\gamma RIIb$ could be manipulated independently. This ability may complement the other

15 required functions (as indicated by the ability to bind protein A) in increasing the therapeutic potential of the binding molecule.

Preferably the effector domain is itself derived from a

20 human immunoglobulin constant region, more preferably an IgG C-domain.

Preferably the comprised amino acid sequence is substantially homologous to the C_H2 sequence (i.e.

25 approximately residues 231-340) from human IgG1, G2 or G4, having the modified amino acids discussed above.

The most preferred C_H2 sequences are shown in Fig 17 and are designated G1Δab ('Seq ID NO 1') and G2Δa ('Seq ID NO

30 2' respectively).

Any of these sequences may be combined with (e.g run contiguously with) natural or modified C_H3, plus optionally C_H1, sequences in the molecules of the present

35 invention.

However it will be appreciated by those skilled in the

art that there is no requirement that other portions of
the effector domain comprise natural sequences - in
particular it may be desirable to combine the sequence
modifications disclosed herein with others, for instance
5   selected from the literature, provided only that the
required activities are retained. The skilled person
will appreciate that binding molecules comprising such
additionally-modified (e.g by way of amino acid addition,
insertion, deletion or substitution) effector domains
10   fall within the scope of the present invention.

The binding domain and target molecule

The peptide molecule comprises a binding domain capable
15   of binding a target molecule.

The binding domain will have an ability to interact with
a target molecule which will preferably be another
polypeptide, but may be any target (e.g. carbohydrate,
20   lipid (such as phospholipid) or nucleic acid). Preferably
the interaction will be specific. The binding domain may
derive from the same source or a different source to the
effector domain.

25   For instance, while the effector domain will generally
derive from an antibody, the binding domain may derive
from any molecule with specificity for another molecule
e.g. an enzyme, a hormone, a receptor (cell-bound or
circulating) a cytokine or an antigen (which specifically
30   binds an antibody).

Preferably, it comprises all or part of an antibody or a
derivative thereof, particularly a natural or modified
variable domain of an antibody. Thus a binding molecule
35   according to the present invention may provide a rodent
or *camelidae* (see WO 94/25591) originating antibody
binding domain and a human immunoglobulin heavy chain as

discussed above.

The binding molecule may comprise more than one
polypeptide chain in association e.g. covalent or
otherwise (e.g. hydrophic interaction, ionic interaction,
or linked via sulphide bridges). For instance it may
comprise a light chain in conjunction with a heavy chain
comprises the effector domain. Any appropriate light
chain may be used e.g. the most common kappa light chain
allotype is Km(3) in the general population. Therefore
it may be desirable to utilise this common kappa light
chain allotype, as relatively few members of the
population would see it as foreign.

Typically the target will be an antigen present on a
cell.

This may be selected as being a therapeutic target,
whereby it is desired to bind it with a molecule having
the properties discussed above, for instance to compete
with or displace undesirable antibodies from it.
Alternatively it may be desirable per se to bind the
target molecule, without causing cell mediated
destruction, antibody triggered inflammation or
complement lysis. Equally the effector domain may
function primarily in mediating transport and/or improved
serum half life - in such cases the binding domain and
target molecule may be any system which would benefit
from these qualities.

Examples include:

1)   Alloimmune disorders such a haemolytic disease of
      the newborn (HDN) where maternal IgG antibodies
      directed against the RhD antigen on red cells of the
      foetus, cross the placenta and cause lysis in utero.

2)   The similar disease, feto-maternal alloimmune
     thrombocytopenia (FAIT), where maternal IgG
     antibodies are directed against alloantigens of the
     Human Platelet Antigen (HPA) systems on the

5   platelets of the fetus, cross the placenta and cause
     platelet destruction in utero. After birth this
     disease is manifested as neonatal alloimmune
     thrombocytopenia (NAITP).

10   An example binding domain could be derived from an anti-
     HPA-1a antibody (see Burrows et al (1993) N Engl J Med
     329, 1463-1466).

3)   Autoimmune disorders where a patient makes

15   antibodies against their own cells or tissues such
     as autoimmune vasculitis where antibodies are made
     against neutrophil antigens.

4)   Monoclonal antibodies are used sometimes to block

20   cell functions, eg OKT3 is used to immunosuppress T-
     cells b blocking the T-cell receptor and CD18
     antibodies are used to prevent cell-cell adhesion
     through the integrin molecules. However the binding
     of the Fc to Fc receptors can trigger serious side

25   effects through stimulating cytokine release and
     inflammation.

5)   Antibody Fc regions are sometimes attached to other
     recombinant proteins to give fusion molecules with

30   prolonged biological half-lives. Thus TNF receptor
     has been attached to human IgG4 Fc to form a
     molecule which inhibits the effects of soluble TNF,
     and CTLA4 has been made as a fusion protein with IgG
     Fc and used to block signalling through the B7

35   coeceptor (a ligand for CTLA4) molecule on cell
     surfaces. However again cytokine triggering by the

Fc of the fusion protein is undesirable.

6) Some antibodies are made against inappropriate
    foreign antigens and trigger strong side effects, eg
5   the production of IgE against pollen and house dust
    mite which can result in allergy and asthma.

V domains, or other binding regions, appropriate to the
types of application discussed above will be well known
10 to those skilled in the art.

Thus a binding molecule which did not bind to Fc
receptors and trigger killing, and did not activate
complement, but which did bind to a target molecule,
15 could be used in all of the above examples to minimise
any side effects. Specifically, such a 'blocking'
antibody could be introduced in situations 1-3 and 6
above and prevent the undesirable destruction by the
naturally occurring antibodies. The same blocking type
20 Fc regions would be the Fc regions of choice to use for
recombinant antibodies such as the CD3 or 4CD18
antibodies in 4 above or as the Fc for fusions in 5
above.

25 The binding and effector domains may be combined in by
any suitable method. For instance domains may be linked
covalently through side chains. Alternatively,
sulphydryl groups generated by the chemical reduction of
cysteine residues have been used to cross-link antibody
30 domains (Rhind, S K (1990) EP 0385601 Cross-linked
antibodies and processes for their preparation).
Finally, chemical modification of carbohydrate groups has
been used to generate reactive groups for cross-linking
purposes. These methods are standard techniques
35 available to those skilled in the art. They may be
particularly applicable in embodiments wherein the
binding polypeptde contains non-protein portions or

groups.

Generally it may be more appropriate to use recombinant techniques to express the binding molecule in the form of a fusion protein. Methods and materials employing this approach form further aspects of the present invention, as set out below.

Nucleic acids

In one aspect of the present invention there is disclosed a nucleic acid encoding a binding molecule as described above.

Nucleic acid according to the present invention may include cDNA, RNA, genomic DNA (including introns) and modified nucleic acids or nucleic acid analogs (e.g. peptide nucleic acid). Where a DNA sequence is specified, e.g. with reference to a Figure, unless context requires otherwise the RNA equivalent, with U substituted for T where it occurs, is encompassed.

Nucleic acid molecules according to the present invention may be provided isolated and/or purified from their natural environment, in substantially pure or homogeneous form, or free or substantially free of other nucleic acids of the species of origin. Where used herein, the term "isolated" encompasses all of these possibilities.

The nucleic acid molecules may be wholly or partially synthetic. In particular they may be recombinant in that nucleic acid sequences which are not found together in nature (do not run contiguously) have been ligated or otherwise combined artificially. Alternatively they may have been synthesised directly e.g. using an automated synthesiser.

In a further aspect there is disclosed a nucleic construct, e.g. a replicable vector, comprising the nucleic acid sequence.

5

A vector including nucleic acid according to the present invention need not include a promoter or other regulatory sequence, particularly if the vector is to be used to introduce the nucleic acid into cells for recombination into the genome.

10

Preferably the nucleic acid in the vector is under the control of, and operably linked to, an appropriate promoter or other regulatory elements for transcription in a host cell such as a microbial, (e.g. bacterial, yeast, filamentous fungal) or eucaryotic (e.g. insect, plant, mammalian) cell.

15

Particularly, the vector may contain a gene (e.g. *gpt*) to allow selection in a host or of a host cell, and one or more enhancers appropriate to the host.

20

The vector may be a bi-functional expression vector which functions in multiple hosts. In the case of genomic DNA, this may contain its own promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

25

By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). The promoter may optionally be an inducible promoter.

30

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented

35

for transcription to be initiated from the promoter.
DNA operably linked to a promoter is "under
transcriptional initiation regulation" of the promoter.

Thus this aspect of the invention provides a gene
construct, preferably a replicable vector, comprising a
promoter operatively linked to a nucleotide sequence
provided by the present invention.

Generally speaking, those skilled in the art are well
able to construct vectors and design protocols for
recombinant gene expression. Suitable vectors can be
chosen or constructed, containing appropriate regulatory
sequences, including promoter sequences, terminator
fragments, polyadenylation sequences, enhancer sequences,
marker genes and other sequences as appropriate. For
further details see, for example, *Molecular Cloning: a
Laboratory Manual*: 2nd edition, Sambrook *et al*, 1989,
Cold Spring Harbor Laboratory Press.

Many known techniques and protocols for manipulation of
nucleic acid, for example in preparation of nucleic acid
constructs, mutagenesis, sequencing, introduction of DNA
into cells and gene expression, and analysis of proteins,
are described in detail in *Current Protocols in Molecular
Biology*, Second Edition, Ausubel et al. eds., John Wiley
& Sons, 1992. The disclosures of Sambrook et al. and
Ausubel et al. are incorporated herein by reference.

Host cells & methods

Also embraced by the present invention are cells
transformed by expression vectors defined above. Also
provided are cell cultures (preferably rodent) and
products of cell cultures containing the binding
molecules.

- 18 -

Also provided are methods of making binding molecules
according to the present invention comprising:
(i) combining a nucleic acid encoding a binding domain
with a a nucleic acid encoding an effector domain to form
5   a nucleic acid construct;
(ii) causing or allowing the expression of the construct
in a suitable host cell.

Combination, to produce a construct, can be by any
10   convenient method known to those skilled in the art, for
instance by ligation of fragments (e.g. restriction
fragments) or using different templates in one or more
amplification steps e.g. using PCR.

15   Methods of producing antibodies (and hence binding
domains) include immunising a mammal (e.g. human, mouse,
rat, rabbit, horse, goat, sheep, camel or monkey) with a
suitable target protein or a fragment thereof.
Antibodies may be obtained from immunised animals using
20   any of a variety of techniques known in the art, and
might be screened, preferably using binding of antibody
to antigen of interest.

For instance, Western blotting techniques or
25   immunoprecipitation may be used (Armitage et al, 1992,
Nature 357: 80-82).

Cloning and expression of Chimaeric antibodies is
described in EP-A-0120694 and EP-A-0125023.
30

The nucleic acid encoding the effector domain can be
generated, in the light of the present disclosure, by
site directed mutagenesis, for instance by methods
disclosed herein or in the published art (see e.g. WO
35   92/16562 or WO 95/05468 both of Lynxvale Ltd).

Other aspects

Also provided is use of the binding molecules of the present invention to prevent, inhibit, or otherwise interfere with the binding of a second binding molecule to a target molecule. This may involve competing with, or displacing, an antibody from a therapeutically relevant target antigen or cell.

5

The present invention also provides a reagent which comprises a binding molecule as above, whether produced recombinantly or otherwise.

10

The present invention also provides a pharmaceutical preparation which comprises a binding molecule as above, plus a pharmaceutically acceptable carrier.

15

The present invention also provides a method of treating patient which comprises administering a pharmaceutical preparation as above to the patient, or to a sample (e.g. a blood sample) removed form that patient, which is subsequently returned to the patient. Particularly a method of treatment for the following diseases: Graft-vs-host disease; host-vs-graft disease; organ transplant rejection; bone-marrow transplant rejection; autoimmunity; allergy; chronic or acute inflammatory diseases.

20

25

The present invention also provides a method of treating patient which comprises causing or allowing the expression of a nucleic acid encoding a binding molecule as described above, whereby the binding molecule exerts its effects *in vivo* in the patient. Generally the expression will occur in the patient, or in certain specialised circumstances where the patient is an unborn infant, in the mother of the patient.

30

35

Also provided is the use of a binding molecule as above in the preparation of a pharmaceutical to modify an

- 20 -

immune response, particularly a pharmaceutical for the
treatment of the diseases discussed above.

In order that the present invention is more fully
5   understood embodiments will now be described in more
detail, by way of example only, and not by way of
limitation. Other embodiments falling within the scope
of the invention may occur to those skilled in the art in
the light of these.

10

FIGURES

Figure 1
Rosetting of FcγRI-bearing cells by RBC coated with Fog-1
15   antibodies. R₂R₂ RBC were coated with Fog-1 antibodies at
a range of antibody concentrations and incubated with
B2KA cells growing in a 96-well plate. Excess RBC were
removed and the percentage of B2KA cells with rosettes of
RBC determined for each well. For the mutants Fog-1
20   G1Δb, G1Δc, G1Δab, G1Δac, G2Δa, G4Δb and G4Δc, as for G2
(shown), there was no association between B2KA cells and
RBC for any of the coating concentrations.

Figure 2
25   Fluorescent staining of FcγRI-bearing cells. FcγRI
transfectant cell lines, B2KA(a and b) and 3T3+FcγRI+γ-
chain (c and d) were incubated sequentially with
antibodies of the CAMPATH-1 (a and c) or Fog-1 (b and d)
series, biotinylated anti-human κ antibodies and
30   ExtrAvidin-FITC. The fluorescence intensities were
measured for 10000 events and the geometric mean channel
of fluorescence plotted.

Figure 3
35   Histogram representation of fluorescently stained FcγRI-
bearing cells. B2KA cells were stained as in Figure 2
using 100 μg/ml antibodies from the CAMPATH-1 series.

The histogram plots showing the number of cells falling
in each fluorescence channel were overlaid for
representative antibodies.

5   Figure 4
CL response of human monocytes to RBC sensitized with
Fog-1 series of antibodies. R_1R_1 RBC were coated with
antibodies over a range of concentrations. The number of
antibody molecules bound per cell and the CL response of
10   moncytes to the RBC was determined for each sample as
described.

Figure 5
Inhibition of CL due to Fog-1 G1 by other Fog-1
15   antibodies. RBC were sensitized with 2 μg/ml Fog-1 G1
and different concentrations of the Fog-1 Ab indicated.
These Ab gave a low CL response in Figure 4. The CL
response of monocytes was measured. The response due to
2 μg/ml G1 alone is taken as 100%.

20

Figure 6
Inhibition of CL response to clinical sera by Fog-1 G2Δa.
RBC were sensitized with a constant amount of Fog-1 G1
(20 μg/ml) or clinically relevant sera and different
25   amounts of Fog-1 G2Δa. 100% response was achieved with a
standard amount of BRAD 5. In the absence of Fog-1 G2Δa,
the % responses were G1: 150%, sera A: 142%, sera B:
265%, sera C: 200%, sera D: 163%, sera E: 94%, anti-C+D
sera: 259% and anti-K sera: 119%.

30

Figure 7
Complement lysis mediated by CAMPATH-1 series of
antibodies. Human PBMC were labelled with ^{51}Cr and
incubated with the antibodies in the presence of serum as
35   a source of complement. The % specific Cr release is
plotted as a measure of lysis occurring.

<u>Figure 8</u>
Inhibition by CAMPATH-1 G2Δa of complement lysis mediated
by CAMPATH-1 G1. Complement lysis was carried out as in
Figure 7 but the samples contained a constant amount
5   (6.25 μg/ml final concentration) of CAMPATH-1 G1 and
increasing quantities of CAMPATH-1 G2Δa.

<u>Figure 9</u>
ADCC mediated by CAMPATH-1 series of antibodies. Human
10   PBMC were labelled with ^{51}Cr and incubated with antibody.
After washing, the cells were incubated with further
PBMC, acting as effector cells, in an effector:target
ratio of 20:1. The % specific Cr release is plotted as a
measure of lysis occurring.
15

<u>Figure 10</u>
ADCC of RhD$^+$ RBC mediated by Fog-1 series of antibodies

<u>Figure 11</u>
20   Inhibition by Fog-1 antibodies of the ADCC of RhD$^+$ RBC
mediated by Fog-1 G1 at 2 ng/mg

<u>Figure 12</u>
Inhibition by Fog-1 antibodies of the ADCC of RhD$^+$ RBC
25   mediated by polyclonal anti-RhD at 3 ng/mg

<u>Figure 13</u>
Fluorescent staining of FcγRIIa 131H/H-bearing cells.
Cells of the transfectant line 3T6+FcγRIIa 131H/H were
30   incubated with the Fog-1 antibodies complexed with goat
F(ab')$_2$ anti-human κ and then with FITC-conjugated donkey
anti-goat IgG. The fluorescence intensities were
measured for 10000 events and the geometric mean channel
of fluorescence plotted.
35

<u>Figure 14</u>
Fluorescent staining of FcγRIIb1*-bearing cells. The

experiment was carried out as in Figure 14 using the
transfectant line 3T6+FcγRIIb1*.

Figure 15
This shows Table 1, which compares the mutations made to
wildtype G1, G2 and G4 sequences.

Figure 16
This shows Table 2, which is a summary of antibody
activities.

Figure 17
This shows the Sequences of certain modified and wild-
type C_H2 sequences, including those designated G1Δab ('Seq
ID NO 1') and G2Δa ('Seq ID NO 2' respectively).

EXAMPLES

General Materials and Methods

Construction of expression vectors

The starting point for the IgG1 constant region was the
human IgG1 constant region gene of allotype G1m(1,17) in
a version of the vector M13tg131 which contains a modifed
polylinker (Clark, M. R.:WO 92/16562). The 2.3kb IgG1
insert thus has a *Bam*HI site at the 5' end and contains a
*Hind*III site adjacent to the *Bam*HI site. At the 3' end,
downstream of the polyadenylation signal, the following
sites occur in the order 5' to 3': *Sph*I, *Not*I, *Bgl*II,
*Bam*HI. The human IgG2 constant region gene had been
obtained as a *Hind*III-*Sph*I fragment in M13tg131 and the
*Hind*III site had been destroyed by digesting with
*Hind*III, filling in the overhanging ends and ligating the
ends together again. The *Sal*I-*Sph*I fragment of this
vector was cloned to replace the equivalent fragment in
the IgG1 vector described above. The human IgG4 constant

region gene had been obtained as a *HindIII-SmaI* fragment in M13tg131 and the *HindIII* site destroyed. The *SmaI* site occurs between the 3' end of the CH3 exon and the polyadenylation site so the polyadenylation site was

5   restored by adding the *SmaI* fragment from the IgG1 vector, which comprises DNA from between the equivalent *SmaI* site in the IgG1 gene and the *SmaI* site downstream of the gene in the polylinker.

10   The first procedure was to introduce an *XbaI* restriction site between the CH1 and hinge exons, a *XhoI* site between the hinge and CH2 exons and a *KpnI* site between the CH2 and CH3 exons in order to facilitate exchange of mutant exon sequences. This was similar to the manipulation of

15   IgG1 and IgG4 genes carried out previously (Greenwood, J., Clark, M. and Waldmann, H. (1993) Structural motifs involved in human IgG antibody effector functions. Eur. J. Immunol. 23, 1098-1104)

20   To provide the template DNAs, *E. coli* RZ1032 was infected with the M13 described above and ssDNA prepared. The strain is *dut⁻ung⁻* so the ssDNA produced should contain some uridine in place of thymidine.

25   The oligonucleotides used to introduce the mutations were:
between the hinge and CH2 exons
MO10   5' GGA TGC AGG CTA <u>CTC GAG</u> GGC ACC TG 3'
between the CH2 and CH3 exons

30   MO11   5' TGT CCA TGT GGC CCT <u>GGT ACC</u> CCA CGG GT 3'
between the CH1 and hinge exons
MO12   5' GAG CCT GCT TCC <u>TCT AGA</u> CAC CCT CCC T 3'
Restriction sites are underlined.

35   The oligonucleotides were phosphorylated in 50 μl reactions containing 25 pmol oligonucleotide and 5u T4 polynucleotide kinase (nbl) in 70 mM Tris HCl pH7.6, 10

mM MgCl$_2$, 100 mM KCl, 5 mM DTT, 0.5 mg/ml BSA, 1 mM ATP.
Reactions were incubated at 37C for 1h and heated at 70C
for 5 min.

5   To anneal the mutagenic oligonucleotides to the template
     DNA, 500 ng uridine-containing DNA and 1 pmol each
     phosphorylated oligonucleotide were incubated in 20 μl
     of 40 mM Tris HCl pH7.5, 20 mM MgCl$_2$, 50 mM NaCl at 80C
     for 5 min and allowed to cool slowly to 37C. The volume
10   was increased to 30 μl with the same buffer and DTT added
     to 7 mM, ATP to 1 mM and dATP, dCTP, dGTP and dTTP each
     to 250 μM. 5 u T7 DNA polymerase (unmodified, United
     States Biochemical) and 0.5 u T4 DNA ligase (Gibco BRL)
     were added and the reaction incubated at room temperature
15   for 16 h to synthesise the mutant strand. The DNA was
     ethanol precipitated, dissolved 50 μl of 20 mM Tris HCl
     pH8.0, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA and 1 u uracil
     DNA glycosylase (New England Biolabs) added. After
     incubating at 37C for 2 h, 50 μl 400 mM NaOH was added
20   and the reaction left at room temperature for 5 min to
     fragment the template strand of DNA. The DNA was ethanol
     precipitated, dissolved in H$_2$O and transformed into *E.
     coli* TG1. Replicative form (RF) DNA was made for a
     selection of the resultant M13 clones and digested to
25   find clones which contained the required *Xba*I, *Xho*I and
     *Kpn*I restriction sites. Suitable clones were obtained
     for the IgG1 and 4 vectors but MO12 appeared to be
     misannealing in the IgG2 vector so the mutagenesis was
     repeated for IgG2 without this oligonucleotide as the
30   site between the CH1 and hinge exons was not necessary
     for these experiments. For each vector, the DNA
     sequences of the exons were confirmed by sequencing.

     The changes in CH2 at amino acid positions 327, 330 and
35   331 (Δa mutation) were to be introduced using the
     oligonucleotides:-
     MO22BACK (coding strand):

5' TCT CCA ACA AAG GCC TCC CGT CCT CCA TCG AGA AAA 3'

MO22 (complementary strand):

5' TTT TCT CGA TGG AGG ACG GGA GGC CTT TGT TGG AGA 3'

The changes in CH2 at positions 233 to 236 (Δb and Δc mutation) were to be introduced using the oligonucleotides:-

MO7BACK (coding strand and encoding Δc mutation):

5' TCC TCA GCA CCT CCA GTC GCG GGG GGA CCG TCA GTC 3'

MO21 (complementary strand and encoding Δb mutation):

5' GAC TGA CGG TCC CGC GAC TGG AGG TGC TGA GGA 3'

The mutations were to be introduce by overlap extension PCR which also required the oligonucleotides MO11 and MO10BACK:

5' CAG GTG CCC TCG AGT AGC CTG CAT CC 3'

XhoI restriction site is underlined.

For the Δa mutation, the first set of PCRs used IgG1 and IgG2 templates amplified with MO22 and MO10BACK and with MO22BACK and MO11. For the Δb and Δc mutations, the first set of PCRs used IgG1 and IgG4 templates with MO21 and MO10BACK and with MO7BACK and MO11. In the final product, DNAs originating from a strand primed with MO21 would have the Δb mutation and those originating from MO22BACK would carry the Δc mutation. Each PCR contained about 30 ng M13tg131+constant region ssDNA, 25 pmol each oligonucleotide and 1 u Pwo DNA polymerase (Boehringer Mannheim) in 50 ul of 10 mM Tris HCl, pH8.85, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 250 μM each dATG, dCTP, dGTP and dTTP. The reactions were subjected to 14 cycles of 94C, 30 s; 50C, 30 s; 72C, 60 s, followed by 72C, 5 min to end. Bands representing product DNAs of the expected sizes were excised from low melting point agarose and melted in 100 μl H_2O. For each mutation, the two initial PCR products were joined together by overlap extension PCR. About 4 μl total of the melted gel slices, such the intial PCR products were in equimolar amounts, were mixed with 25 pmol each MO10BACK and MO11 and other components

as above. The PCR was carried out over 18 cycles as
above except that the annealing temperature was reduced
from 50C to 48C. The products obtained, which contained
the entire CH2 exon, were purified and digested with *Xho*I
and *Kpn*I. The RF DNAs of the mutated M13tg131+constant
region vectors, containing the extra restriction sites as
described above, were digested with *Xho*I and *Kpn*I to
remove the existing CH2 DNAs and the mutant CH2 regions
ligated in. The DNA samples were transformed into *E.
coli* TG1. DNA of representative clones was sequenced to
identify correctly mutated constant regions.

In order to obtain IgG1 vectors with both Δa and Δb or
Δc, DNA, representing a Δa mutant, was used as the
template for a second round of PCRs to introduce the Δb
and Δc mutations as described above.

The IgG1, 2 and 4 wild type and mutated constant region
genes were each excised from RF DNA as a *Bam*HI - *Not*I
fragment and cloned into the modified CAMPATH Hu4VH
HuIgG1 pSVgpt vector (Clark, M. R.: Lynxvale Binding
Molecules as above) to replace the existing constant
region. The resulting vectors were designated
pSVgptCAMPATHHu4VHHuIgG1Δa, etc. The vector also contains
the *gpt* gene to allow selection in mammalian cells, the
murine immunoglobulin heavy chain enhancer and the
CAMPATH-1 Hu4VH variable region DNA so that it carries a
complete heavy chain gene which can be expressed in
mammalian cells. The CAMPATH-1 humanised light chain
gene exists in the expression vector CAMPATH HuVL pSVneo
(Reichmann, L., Clark, M. R., Waldmann, H. and Winter, G.
(1988) Nature 332, 323-327).

The Fog1 variable region DNAs (Bye, J. M., Carter, C.,
Cui, Y., Gorick, B. D., Songsivilai, S., Winter, G.,
Hughes-Jones, N. C. and Marks, J. D. (1992) Germline
variable region gene segment derivation of human

monoclonal anti-Rh(D) antibodies. J. Clin. Invest. 90, 2481-2490) were obtained in the vector pHEN1. They were amplified by PCR, using the oligonucleotides:-

FOG1VHBACK   5' TCC ACA GGT GTC CAC TCC CAG GTG CAT CTA CAG CAG 3'

FOG1VHFOR   5' GAG GTT GTA AGG ACT CAC CTG AGG AGA CGG TGA CCG T 3'

FOG1VKBACK   5' TCC ACA GGT GTC CAC TCC GAC ATC CAG ATG ACC CAG 3'

FOG1VKFOR   5' GAG GTT GTA AGG ACT CAC GTT TGA TCT CCA GCT TGG T 3'

The 5' portion of the insert in the vector M13VHPCR1 (Orlandi, R., Gussow, D. H., Jones, P. T. and Winter, G. (1989) Proc. Natl. Acad. Sci. USA 86, 3833), comprising the promoter and DNA encoding the signal peptide was amplified using the universal M13 reverse primer and VO3:

5' GGA GTG GAC ACC TGT GGA GA 3'

DNA, 3' of the V_H in M13VHPCR1 and representing the 5' end of the V_H-C_H intron, was obtained by PCR using the universal M13 -40 primer and VO4:

5' GTG AGT CCT TAC AAC CTC TC 3'

These two segments of DNA were joined sequentially to both the Fog-1 V_H and Fog-1 V_κ amplified DNA by overlap extension PCR as described above. The *Bam*HI restriction site internal to the Fog-1 V_H was deleted by the same method using oligonucleotides which removed the recognition site without changing the amino acids encoded. The complete PCR products were cloned into M13mp19 as *Hin*dIII - *Bam*HI fragments and their DNA sequences confirmed.

The *Hin*dIII - *Bam*HI fragment containing the Fog-1 V_H was used to replace the fragment containing the CAMPATH-1 V_H in the pSVgpt vectors described above, giving expression vectors designated pSVgptFog1VHHuIgG2, etc. For the IgG1 vectors, the extra *Hin*dIII restriction site at the 5' end of the constant region DNAs meant that it was not

possible to simply exchange the *Hind*III - *Bam*HI variable region fragment. Instead, the relevant pSVgptCAMPATHHu4VHHuIgG1 vectors were digested with *Hind*III. Linkers, designed to delete the *Hind*III site

5   and add a *Bam*HI site, were ligated onto the cut ends. The DNAs were then digested with *Bam*HI and *Not*I so that the constant regions could be isolated and these were cloned into pSVgptFog1VHHuIgG2 to replace the IgG2 constant region.

10

The *Hind*III - *Bam*HI fragment containing the Fog-1 V_κ was transferred to the vector pSV*hyg*-HuCK (Orlandi *et al.*, 1989) which already contains the murine immunoglobulin heavy chain enhancer and the human κ constant region

15   gene. The resulting expression vector was called pSVhygFog1VKHuCK.

Production of antibodies

20   10 μg of each heavy chain expression vector and 20 μg of the relevant light chain expression vector were linearised by digestion with *Pvu*I and combined in 50 μl of H_2O. Cells of the non-secreting rat myeloma line, YB2/0, were grown to semi-confluency in Iscove's modified

25   Dulbecco's medium (IMDM) with 5% foetal bovine serum (FBS). 10^7 cells were collected by centrifugation, resuspended in 0.5 ml medium and transferred to a GenePulser cuvette (BioRad). The DNA was added and the mixture incubated on ice for 5 min. The cells were given

30   one pulse of 960 μF/170 V and returned to ice for 15 min before being placed in a flask in 20 ml IMDM + 10% FBS. They were incubated at 37C, 5% CO_2 in a humidified atmosphere. After 24 h, the volume was doubled and the medium made selective by addition of mycophenolic acid to

35   0.8 μg/ml and xanthine to 250 μg/ml. The cells were aliquotted over two 96-well plates. About 18 d after selection was applied, colonies were visible and the

supernatants were assayed for the presence of IgG by ELISA. Briefly, microtitre-plate wells were coated with goat anti-human IgG, Fc-specific antibodies (Sigma) and then incubated with 5-fold dilutions of the supernatants.

5   Bound antibody was detected by incubating with HRPO-conjugated goat anti-human κ antibodies (Seralab) and developing the assay with o-phenylenediamine substrate. Cells from wells containing the highest amounts of antibody were expanded and stocks cryopreserved.

10

The cell line secreting the highest amounts of Ab was expanded to 500 ml in IMDM + 2% FBS to provide saturated supernatant for antibody purification. The supernatant was cleared by centrifugation and made 0.1 M Tris HCl

15   pH8.0. Protein A-agarose (Sigma) was added and the mixture stirred at 4C for 16 h. The agarose beads were collected into a column and washed with 0.1 M Tris HCl pH8.0, followed by 10 mM Tris HCl pH8.0. The antibody was eluted with 1 ml aliquots of 0.1 M glycine pH3.0 into

20   100 μl samples of 1 M Tris HCl pH8.0 and the fractions containing significant amounts of protein were identified from A_{280nm} readings. These fractions were dialysed against PBS, filter-sterilised and the A_{280nm} remeasured to give the approximate antibody concentration

25   (concentration=A_{280nm} x 0.714 mg/ml).

The purity and integrity of the antibodies were established by reducing SDS-PAGE, using 12.5% acrylamide. The concentrations were checked in an ELISA which used

30   goat anti-human κ antibodies (Seralab) as the capture reagent and biotinylated goat anti-human κ antibodies (Sigma) followed by ExtrAvidin-HRPO (Sigma) for detection. This meant that the nature of the heavy chain was unlikely to influence the level of binding obtained.

35

Rosetting of FcγRI transfectants

Washed R_2R_2 RBC were incubated with Ab samples in 100 μl
PBS in 96-well plates at room temperature for 1 h.   The
RBC were washed three times, resuspended in PBS and added
to transfectants expressing FcγRI cDNA, B2KA (S. Gorman

5   and G. Hale, unpublished) growing in a 96-well plate.
Following a 40 min incubation at 37C, the supernatant was
discarded and the wells washed once with PBS to remove
excess RBC.   The wells were refilled with PBS and the
percentage of B2KA cells which were rosetted by RBC

10   noted.

Fluorescent staining of FcγR transfectants

Transfectants expressing FcγRI cDNA, B2KA and

15   3T3+FcγRIa+γ-chain (van Urgt, M. J., Heijnen, I. A. F.
M., Capel, P. J. A., Park, S. Y., Ra, C., Saito, T.,
Verbeek, J. S. and van de Winkel, J. G. J. (1996) FcR γ-
chain is essential for both surface expression and
function of human FcγRI (CD64) *in vivo*.   Blood 87, 3593-

20   3599), were obtained as single cell suspensions in
phosphate-buffered saline containing 0.1% (w/v) NaN_3, 0.1%
(w/v) BSA (wash buffer) following treatment with cell
dissiocation buffer (Gibco BRL).   Cells were pelleted at
10^5 cells/well in 96-well plates, resuspended in 100 μl

25   dilutions of the CAMPATH-1 or Fog-1 Ab and incubated on
ice for 30 min.   Cells were washed three times 150
μl/well wash buffer and similarly incubated with 20 μg/ml
biotin-conjugated goat anti-human κ-chain Ab (Sigma) and
then with 20 μg/ml ExtrAvidin-FITC (Sigma).   After the

30   final wash, cells were fixed in 100 μl wash buffer
containing 1%(v/v) formaldehyde.   Surface expression of
FcγRI was confirmed by staining with CD64 mAb (Serotec)
and FITC-conjugated goat and mouse IgG Ab (Sigma).
Fluorescence intensities were measured on a FACScan

35   (Becton Dickinson).

For transfectants bearing FcγRII, 3T6 + FcγRIIa 131H/H

(Warmerdam, P. A. M., van de Winkel, J. G. J., Vlug, A., Westerdaal, N. A. C. and Capel, P. J. A. (1991) A single amino acid in the second Ig-like domain of the human Fcγ receptor II is critical for human IgG2 binding. J. Immunol. **147**, 1338-1343) and 3T6 + FcγRIIb1* (Warmerdam, P. A. M., van den Herik-Oudijk, I. E., Parren, P. W. H. I., Westerdaal, N. A. C., van de Winkel, J. G. J. and Capel, P. J. A.(1993) Int. Immunol. 5, 239-247) the antibodies were complexed before being incubated with the cells. The antibodies were mixed with equimolar amounts of goat F(ab')$_2$ anti-human κ (Seralab) and incubated at 37C for 1 h. The complexes were then mixed with the cells and the assay continued as above except that the detecting antibody was FITC-conjugated donkey anti-goat IgG (Serotec).

Red Cell Sensitization

Group O R$_1$R$_1$ RBC were washed in PBS and resuspended in RPMI + 10% FBS at a final concentration of 5% v/v. 10 μl of cells was added to 50 μl mAb or RPMI/FBS in V-bottom well plates and incubated for 60 min at 37C. In some experiments, the mAb were serially diluted in RPMI/FBS to achieve a range of red cell-bound IgG. In competition experiments, the red cells were sensitized in a mixture of 25 μl competing mAb and 25 μl of wild-type mAb or 25 μl serum containing alloantibodies. After sensitization, cells were washed 4 times with 200_μl volumes of PBS and resuspended in 50 μl RPMI/FBS (final concentration = 1% v/v). In all experiments, an aliquot of cells (E-IgG) was used in the CLT and an aliquot was assayed by flow cytometry to determine the level of red cell-bound IgG.

Chemiluminescence Assay

PBMC were isolated by density gradient centrifugation from EDTA-anticoagulated blood pooled from 6 normal

donors. PBMC were washed 4 times with PBS containing 1% globulin-free BSA and then resuspended at 2×10^6/ml in Hank's Balanced Salt Solution (HBSS) containing 25% RPMI and 2.5% FBS. Aliquots (100 μl) were dispensed into 96 flat-bottomed white opaque plates and incubated for 2 h at 37C in a humidified atmosphere of 5% CO_2 in air. The plates were then placed in a luminometer (Anthos Lucy 1, Labtech International, Uckfield, UK) and 100 μl HBSS containing 4×10^{-4} M luminol (Sigma) and 20 μL E-IgG were added to each well. The CL response was then monitored at 37C for 60 minutes.

Determination of Red Cell Bound IgG

25 μl aliquots of E-IgG were transferred to a V-bottom well plate, washed once with PBS, centrifuged to a pellet and resuspended in 50 μl F(ab)$_2$ FITC-anti-IgG (diluted 1/30 in PBS/1% BSA). After 30 min at room temperature, the cells were washed once with 200 μl PBS/BSA and kept on ice until analysed by flow cytometry (EPICS XL-MCL, Coulter Electronics, Luton, UK). The mean channel fluorescence was recorded.

Mean channel fluorescence was converted to IgG molecules/cell by use of a standard curve which was prepared by adding 100 μl of 5% v/v R_1R_1 cells to 900 μl of serial 2 fold dilutions of human monoclonal IgG1 anti-D (BRAD-5). Sensitized red cells were washed 3 times with PBS/BSA and resuspended to 1% v/v in PBS/BSA. 25 μl aliquots were removed and analysed by flow cytometry as described above. The remaining red cells were counted, centrifuged to a pellet, lysed in a buffer containing Triton X-100 and IgG in lysates was determined by ELISA as described by Kumpel (Kumpel, B.M. (1990). A simple non-isotopic method for the quantitation of red cell-bound immunoglobulin. *Vox Sanguinis*, 59, 34-39). The number of IgG molecules bound per red cell was deduced

from the IgG concentration and the number of red cells from which each lysate was prepared. A standard curve was then plotted comparing fluorescence intensity with the number of IgG molecules bound per red cell.

Complement lysis mediated by CAMPATH-1 series of antibodies

100 ml venous blood from a healthy volunteer was defibrinated and components separated by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia). The serum and mononuclear cell layers were removed to fresh tubes. The cells were diluted into Iscove's modified Dulbecco's medium (IMDM) and collected by centrifugation. The cells were washed twice in IMDM whilst being combined into one pellet which was resuspended in 200 μl IMDM. 900 μCi sodium [^{51}Cr] chromate was added and the cells incubated at 37C for 40 min. 10 ml IMDM was added and the cells pelleted. The cells were washed twice and resuspended in IMDM at approximately 6 x 10^6 cells/ml. 50 μl aliquots of labelled cells were added to antibody samples in 50 μl IMDM in 96-well plate wells. 100 μl retained serum diluted 1:1 with IMDM was added to each well and the plates incubated at 37C for 1 h. The plates were centrifuged and the supernatants were sampled and the relative amounts of ^{51}Cr released were measured in a γ-counter. The level of spontaneous release was obtained from samples were no antibody was added and a measure of the total amount of ^{51}Cr available for release was found from similar samples taken after resuspending the cells. The % specific ^{51}Cr release was calculated from the formula:

(sample counts - spontaneous counts)x 100
(total counts- spontaneous counts)

The means and standard deviations of the triplicate samples were plotted.

For the inhibiton of complement lysis, antibody samples contained a constant amount (6.25 μg/ml final concentration) of CAMPATH-1 G1 and increasing quantities of CAMPATH-1 G2Δa.

5

ADCC mediated by CAMPATH-1 series of antibodies

Peripheral blood mononuclear cells were prepared as described above. After washing, the cells were
10 resuspended in IMDM supplemented with 5% FBS and transferred to flask which had been coated with CD3 antibody. The cells were grown at 37C, 5% CO_2 for three days. 5% of the cells were labelled with ^{51}Cr for use as target cells, washed and resuspended at 6 x 10^5 cells/ml
15 in IMDM + 5% FBS. 50 μl aliquots were added to wells of 96-well plates containing 50 μl samples of antibodies in IMDM + 5% FBS. The target cells and antibodies were incubated at 37C for 1 h, RBC added as carriers and the cells pelleted. The cells were washed twice in IMDM.
20 The remaining mononuclear cells were collected by centrifugation and resuspended at 4 x 10^6 cells/ml in IMDM + 5% FBS and 150 μl added to each well resuspending the target cells in the process. This gives an effector:target ratio of 20:1. The cells were
25 centrifuged gently and placed in a tissue culture incubator for 6 h. Supernatant was sampled and specific ^{51}Cr release determined as described above. The mean values of specific release for the duplicate samples was plotted against the final antibody concentrations.

30

Example 1 - Generation and basic characterisation of antibodies

The mutations chosen to eliminate the effector functions
35 are shown in Table 1 (Fig 15). The Δa mutation made in IgG1 and IgG2 genes introduces the IgG4 residues at positions 327, 330 and 331. Similarly, the IgG2 residues

at positions 233 - 236 were introduced into IgG1 and IgG4 but, since IgG2 has a deletion at 236 where the other subclasses have a glycine residue, the mutation was made omitting (Δb) or including (Δc) G236.

Vectors allowing expression of CAMPATH-1 or Fog-1 V$_H$ DNA in conjunction with the wildtype or mutant constant region genes were cotransfected with the appropriate light chain expression vectors into rat myeloma cells. Stable transfectants were isolated, expanded and Ab purified from the supernatant on protein A-agarose.

CAMPATH-1H was selected as it provides a good targeting system for studying complement and cell mediated lysis *in vitro*.

For the Fog-1 Ab, a precipitate formed after purification but, once this had been removed by filter-sterilisation, no further precipitation was noticed. Ab concentrations were estimated from the absorbance at 280 nm and were adjusted where necessary following an ELISA which measures the relative amounts of κ-chain present. The Ab were subjected to reducing SDS-PAGE. Each sample showed two bands with apparent molecular weights of approximately 25 and 55 kDa which represent the expected sizes of the light and heavy chains. There was no discernible difference in size between the heavy chains of each Ab series but both chains of the Fog-1 Ab appeared to be slightly smaller than their CAMPATH-1 counterparts. For the Ab with CAMPATH-1 specificity, the yield after purification varied from 0.6 to 9 μg/ml supernatant whereas the yield of soluble Fog-1 Ab was between 3 and 20 μg/ml.

The specificities of the two series of Ab were then tested. The CAMPATH-1 Ab were shown to compete with clinical grade CAMPATH-1H in the binding of the anti-

- 37 -

CAMPATH-1 idiotype mAb, YID13.9. The Fog-1 Ab where able
to agglutinate RhD⁺ RBC in the presence of anti-human IgG
Ab as cross-linking reagents. Similarly, the IgG
subclasses of the Fog1 Ab were examined by coating RhD⁺
RBC with the different Ab and looking at the
agglutination pattern using anti-G1m(a), anti-IgG2 or
anti-IgG4 Ab as the cross-linking Ab. The result
indicated that the antibodies were of the correct
subclasses. The agglutination of RhD⁺ RBC by Fog-1 IgG1
and anti-G1m(a), by Fog-1 IgG2 and anti-IgG2 and by Fog-1
IgG4 and anti-IgG4 was then carried out in the presence
of excess Ab from the CAMPATH-1 series. The CAMPATH-1 Ab
were able to inihibit the agglutination, by competing for
the cross-linking reagent, only where they were of the
same subclass as the Fog-1 Ab, thus verifying their
subclasses.

Example 2 -   FcγRI binding

R_2R_2 RBC were coated with the Fog-1 series of Ab over a
range of concentrations and added to FcγRI-expressing
transfectants, B2KA, growing in wells. After incubating
excess RBC were washed away and the percentage of B2KA
cells rosetted by RBC was recorded (Figure 1). For G1
and G1Δa, similar levels of rosetting are achieved, with
the midpoint of the rosetting profile occurring when the
RBC are coated with Ab at about 1 μg/ml, a concentration
at which Fog-1 Ab will be expected to occupy
approximately two-thirds of the RhD sites. Two- to four-
fold higher concentrations of G4 are needed to obtain the
same levels of rosetting. No rosettes were formed when
using RBC coated with any of the other Ab. In the
experiment shown here, the highest coating concentration
tested was 10 μg/ml representing approximately 90%
occupancy of RhD sites but the experiment has been
repeated using coating concentrations of up to 80 μg/ml,
essentially saturating the RhD sites, and still no

- 38 -

rosettes were seen for G2 and the Ab containing the Δb or Δc mutations. This indicates that, even when the RBC are coated with Ab at the maximum density for this antigen, the total of the Fc and receptor interactions is
5 insufficient for rosette formation.

R_2R_2 RBC were coated with a mixture of 1 μg/ml Fog-1 G1 and different amounts of Fog-1 G2Δa or Fog-1 G4Δb and used to rosette B2KA cells. In the absence of the second
10 Ab, Fog-1 G1 coated RBC rosetted 95% of the B2KA cells but addition of 64 μg/ml G2Δa or G4Δb to the coated mixture inhibited the rosetting completely such that no rosettes were visible at all.

15 The binding of Ab from both series to two different cell lines, which express the FcγRI cDNA on their surface, was measured by fluorescent staining. Figure 2 shows representative experiments. The level of surface-expressed FcγRI, as detected using the CD64 Ab, was
20 higher for the 3T3 transfectants than for the B2KA line and this reflects in the higher signals obtained when measuring binding via the Fc. For both series, the G1 and G1Δa Ab bound to the receptor with the same apparent affinity indicating that the mutations at positions 327,
25 330 and 331 did not significantly affect the interaction. The binding of G4 Ab was approximately three-fold lower than that of the G1 and G1Δa Ab. Little binding was seen for the G2 Ab or any of the other mutant Ab, suggesting that the Δb and Δc mutations in IgG1 and IgG4 were
30 sufficient to reduce binding to FcγRI by at least 10^4-fold. Ab containing the Δc mutation, especially G1Δc, showed a small degree of binding to FcγRI at the highest concentrations tested if the level of fluorescence is compared to the background or to the equivalent Ab with
35 the Δb mutation. If the fluorescence intensity histograms are overlaid, as seen in Figure 3 for the highest concentrations of CAMPATH-1 Ab and B2KA cells,

the plots for G1 and G1Δa coincide. There is a clear difference between the histograms for the G1Δb and G1Δc Ab.

5 Example 3 - FcγRI triggering measured by chemiluminescence

In order to measure functional activity through FcγRI/II, the chemiluminescent (CL) response of monocytes to RBC

10 sensitized with Ab from the Fog-1 series was measured and plotted in relation to the number of Ab molecules bound per RBC (Figure 4). A difference between the G1 and G1Δa Ab is seen with higher amounts of Ab but both are give higher responses than the G4 Ab across the range of Ab

15 concentrations. Significant triggering is achieved by the G1Δc Ab and, to a lesser extent, by G1Δac and G4Δc but the other Ab do not give any response.

Ab, which were known to be deficient in the triggering of

20 FcγRI from the previous section, were mixed in increasing concentrations with a constant amount of Fog-1 G1 and used to sensitize RBC. The CL response to the RBC is shown in Figure 5. By comparing the CL response to that obtained when titering G1 alone, it appears that six of

25 the eight Ab inhibit the reaction to an extent which predicted if it is assumed that the mutants displace the active G1 from RBC in proportion to their relative concentrations. For G2, the inhibitory effect is delayed in that about three-fold more G2 is needed to give the

30 same amount of inhibition. G1Δc inhibits to approximately the same extent as the other mutants except that the response is not reduced to zero.

Two papers which have discussed the usefulness of

35 chemiluminescence in predicting the severity of in-vivo pathology are Hadley (1995) Transfusion Medicine Reviews 9:302-313 and Hadley et al (1998) Br J Obstet Gynaecol

105: 231-234.

In these assays a result above 30% chemiluminescence produced by the BRAD-5 monoclonal antibody control would be predictive of *in-vivo* pathology in HDN. Thus those antibodies which can block to levels below 30% should be suitable for therapy.

One of the mutant Ab, Fog-1 G2Δa was tested for its ability to inhibit the CL response to sera containing clinically significant Ab. The sera contained anti-RhD Ab or antiC+D and, in the absence of inhibitor, gave CL responses of greater than 30% on this scale which is indicative of severe haemolytic disease of the newborn and the need for intrauterine transfusions. The sera were mixed with different concentrations of G2Δa, the mixtures used to sensitise RBC and the responses of monocytes measured (Figure 6). The addition of G2Δa Ab reduced the CL signals due to all five anti-RhD sera to below the 30% cut-off. The amount of Ab needed to achieve this varied from 16 - 260 μg/ml, the range presumably reflecting the differing amounts and affinities of anti-RhD Ab in the serum. There are two control sera. The anti-K serum cannot be blocked at all by G2Δa as its reactivity is directed towards a diffferent antigen on the RBC. Only part of the activity of the anti-C+D serum could be inhibited by G2Δa.

Example 4 - Activity in complement lysis

Figure 7 shows that all the mutations made to the G1 and G2 CAMPATH-1 antibodies dramatically reduced their ability to mediate complement lysis. When the assay was carried out using a constant amount of G1 and different amounts of G2Δa (Figure 8), the G2Δa antibody was able to block the killing of PBMC by CAMPATH-1 G1.

<u>Example 5 - Activity in ADCC</u>

The ability to mediate ADCC was measured for the CAMPATH-
1 antibodies on PBMC (Figure 9) and for some of the Fog-1
antibodies on RhD⁺ RBC (Figure 10). Figure 9 shows mixed
abilities of the CAMPATH-1 antibodies in ADCC, with some
of the mutants having very low activities. Figure 10
shows that the three selected mutants were unable to
support any killing of RBC.

The same three mutants were used to try to inhibit the
ADCC of RhD⁺ RBC by Fog-1 G1 (Figure 11) and by a clinical
sample of anti-RhD serum (Figure 12). The figures show
that all three mutants were able to inhibit ADCC when
mixed with the active antibodies prior to RBC
sensitisation but G1Δab and G4Δb were particularly
effective.

<u>Example 6 - FcγRII binding</u>

Figures 13 and 14 show the binding of the Fog-1 series of
antibodies to cells bearing FcγRIIa 131H/H and FcγRIIb1*
respectively. FcγRIIa 131H/H is the allotype of FcγRIIa
to which IgG2 antibodies are expected to bind and,
indeed, G1 and G2 show a strong binding activity.
Addition of the mutations to these two antibodies appears
to give a stepwise reduction in the levels of binding and
the G1Δc and G1Δac have only background levels of binding
as exhibited by the G4 antibodies. All of the antibodies
tested appear to bind to FcγRIIb1*. The negative control
in this experiment was the crosslinking F(ab')₂ alone but
the an aglycosylated antibody gave the same result when
used as the negative control in another experiment.

<u>Example 7 - Production of the anti-HPA-1a antibodies</u>

The V$_H$ and V$_λ$ of the anti-HPA-1a scFv (Griffin,H.M. and

Ouwehand, W.H. (1995) A human monoclonal antibody
specific for the leucine-33 form of the platelet
glycoprotein IIIa from a V gene phage display library.
Blood 86, 4430-4436) were amplified and each attached to
leader sequence from the vector M13VHPCR1 (Orlandi et
al., 1989) by overlap extension PCR as described
previously. DNA, 3' of the V_H in M13VHPCR1 and
representing the 5' end of the V_H-C_H intron, was similarly
joined to the leader/V_HDNA. The product was cloned as a
HindIII- BamHI fragment into IgG1 and IgG2 expression
vector to replace the existing variable region fragment
and to give the vectors pSVgptB2VHHuIgG1 and
pSVgptB2VHHuIgG2.

The leader/ Vλ DNA was joined in frame to the human λ
chain constant region DNA of the Kern Oz allotype
(Rabbitts, T.H. Forster, H. and Matthews, J.G. 1983. Mol.
Biol. Med.1:11), taken from an existing expression vector
(Routledge, E.G., Lloyd, I, Gorman, S.D., Clark, M. and
Waldmann, H. 1991, Eur. J. Immunol. 21:2717). The whole
λ gene was cloned into M13 as a HindIII-BamHI fragment
and the murine heavy chain enhancer from pSVhyg-HuCK
(Orlandi et al., 1989) added 5' of the gene using
adapters so that the whole insert could be transferred to
pSV2neo (Southern, P. J. and Berg. P. 1982. J. Mol. Appl.
Genet. 1:327) as a BamHI fragment. The vector was
designated pSVneoB2VλHuCλ.

The expression vectors were transfected into the rat
myeloma cell line YB2/0, transfectants selected and
antibody purified as described before. These B2IgG1 and
B2IgG2 antibodies can be used as control antibodies.

Once the preferred null constant regions have been
selected, the B2 VH HindIII-BamHI fragment can be

introduced into expression vectors carrying the appropriate constant region genes, replacing the existing variable region fragment. The heavy chain expression vectors can then be co-transfected with pSV*neo*B2VλHuCλ into myeloma cells and the antibodies purified for use.

Example 8 - Therapeutic use of binding molecule

A therapeutic molecule according to the present invention may be used to treat pregnancies complicated by HPA-1a alloimmunisation, for instance by intravenous administration to the mother, thereby relying on placental transfer (e.g. via the FcRn) to provide a therapeutic dose to the fetus.

An alternative is direct administration to the fetus by percutaneous umbilical vessel sampling. This procedure is currently performed in FAIT to deliver transfusions of compatible platelets. Because of the short survival of transfused platelets, the procedure may have to be repeated many times during the course of a pregnancy. It is however hazardous, with a risk of fetal loss of 0.5%/procedure.

However, fetal administration of a therapeutic antibody would have the advantage that a much lower dose is likely to be required, and therefore a combined approach using the molecules of the present invention in conjunction with platelet transfusion may be considered as a first step in therapy. This approach may reduce or eliminate the need for further platelet transfusions before delivery.

Summary

The activities of the antibodies are summarised in Table 2 (Fig 16).   As can be seen, binding molecules have been produced which have reduced ability to bind to FcγRI and to FcγRIIa 131; are unable to trigger monocyte chemiluminescence; cannot mediate complement lysis and are not active in ADCC.

Selected mutants have been shown to be able to inhibit completely the rosetting of FcγRI-bearing cells by Fog-1 G1; the response of monocytes to Fog-1 G1-sensitised RBC; the response of monocytes to polyclonal anti-RhD-sensitised RBC; the killing of PBMC by complement lysis with CAMPATH-1 G1; the killing of RBC by ADCC with Fog-1 G1; the killing of RBC by ADCC with polyclonal anti-RhD serum.

Figure 1

Figure 2

a

b

c

d

(x-axes: antibody concentration, µg/ml; y-axes: mean channel fluorescence)

Legend:
—○— G1   —◇— G1Δa   —□— G1Δb   —△— G1Δc   —✕— G1Δab   —╂— G1Δac

—○— G2   —◇— G2Δa   —●— G4   —■— G4Δb   —▲— G4Δc

2/17

Figure 3

Figure 4

The plot shows % chemiluminescence (y-axis, ranging from -20 to 140) versus antibody molecules/cell (x-axis, ranging from 0 to 30000). Legend:

- o G1
- ◇ G1 Δ a
- □ G1 Δ b
- △ G1 Δ c
- ✕ G1 Δ ab
- + G1 Δ ac
- • G2
- ◇ G2 Δ a
- ● G4
- ■ G4 Δ b
- ▲ G4 Δ c

Figure 5

Legend:
- ■ G1Δ b
- △ G1Δ c
- ✕ G1Δ ab
- + G1Δ ac
- ○ G2
- ◇ G2Δ a
- ■ G4Δ b
- ▲ G4Δ c

Y-axis: % chemiluminescence (0 to 100)
X-axis: inhibitor concentration, μg/ml (0.1 to 1000)

Figure 6

Figure 6

(Legend:)
- G1
- anti-D sera A
- anti-D sera B
- anti-D sera C
- anti-D sera D
- anti-D sera E
- anti-C+D sera
- anti-K sera

Y-axis: % chemiluminescence

X-axis: G2Δa concentration, µg/ml

Figure 7

Legend:
- G1
- G1Δ a
- G1Δ b
- G1Δ c
- G1Δ ab
- G1Δ ac
- G2
- G2Δ a
- G4
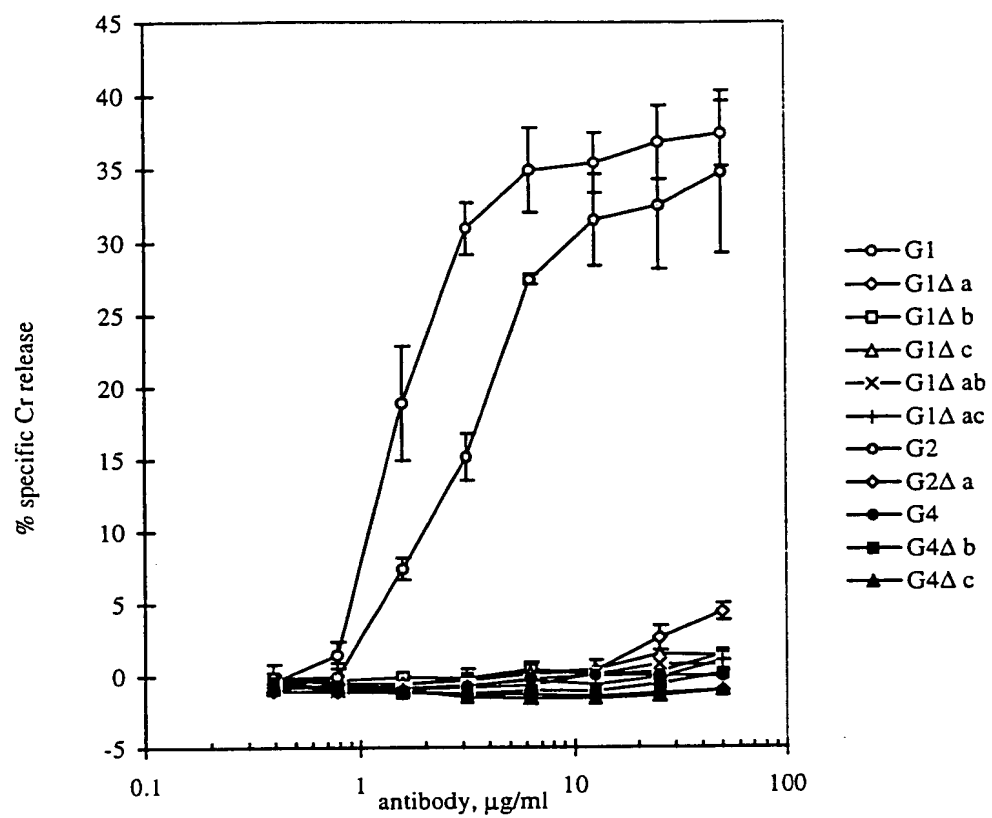- G4Δ b
- G4Δ c

Y-axis: % specific Cr release

X-axis: antibody, μg/ml

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Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Y-axis: % lysis

X-axis: inhibitor antibody concentration, ng/ml

Legend:
—X— G1Δ ab
—○— G2
—◇— G2Δ a
—●— G4
—■— G4Δ b

12117
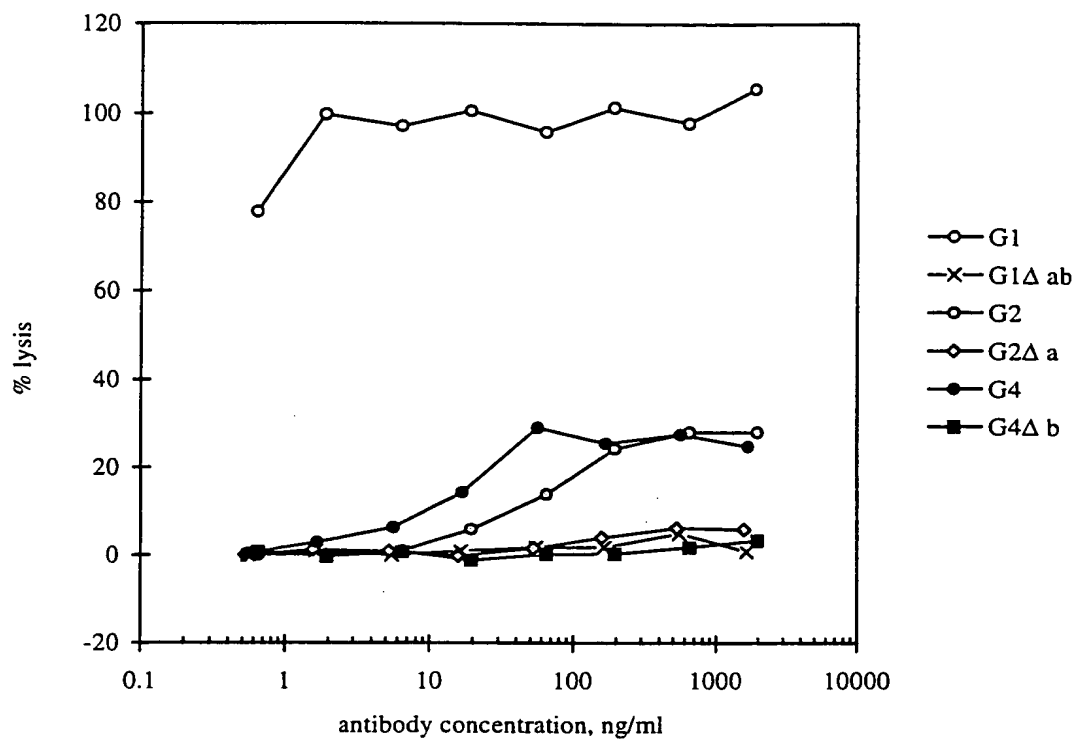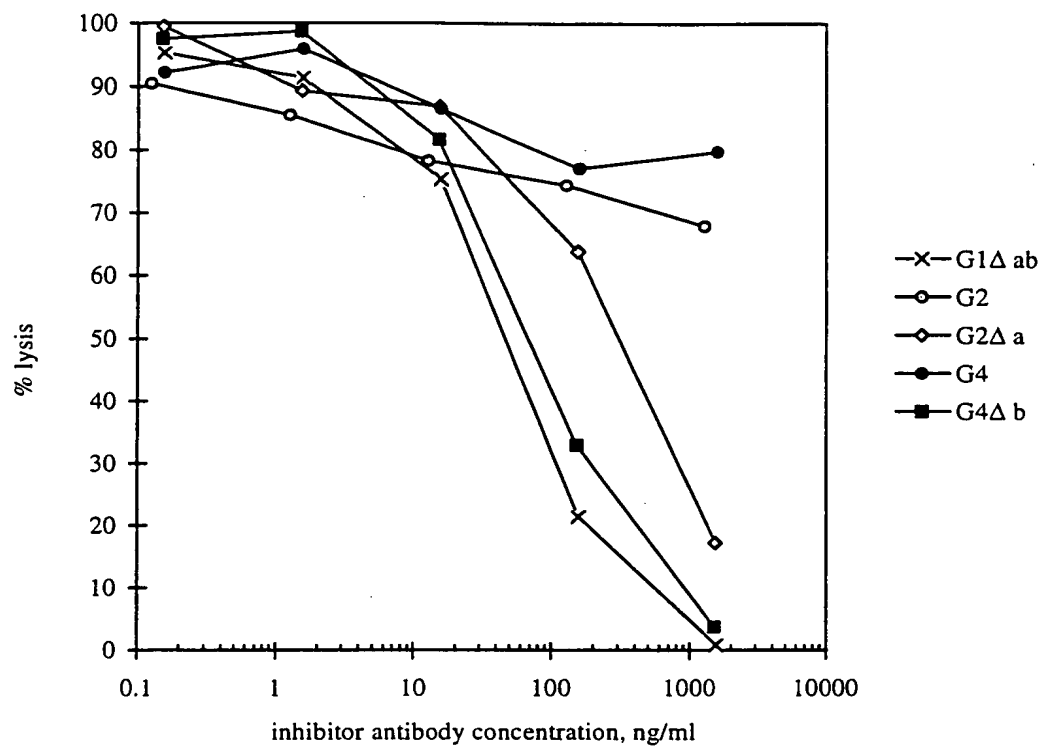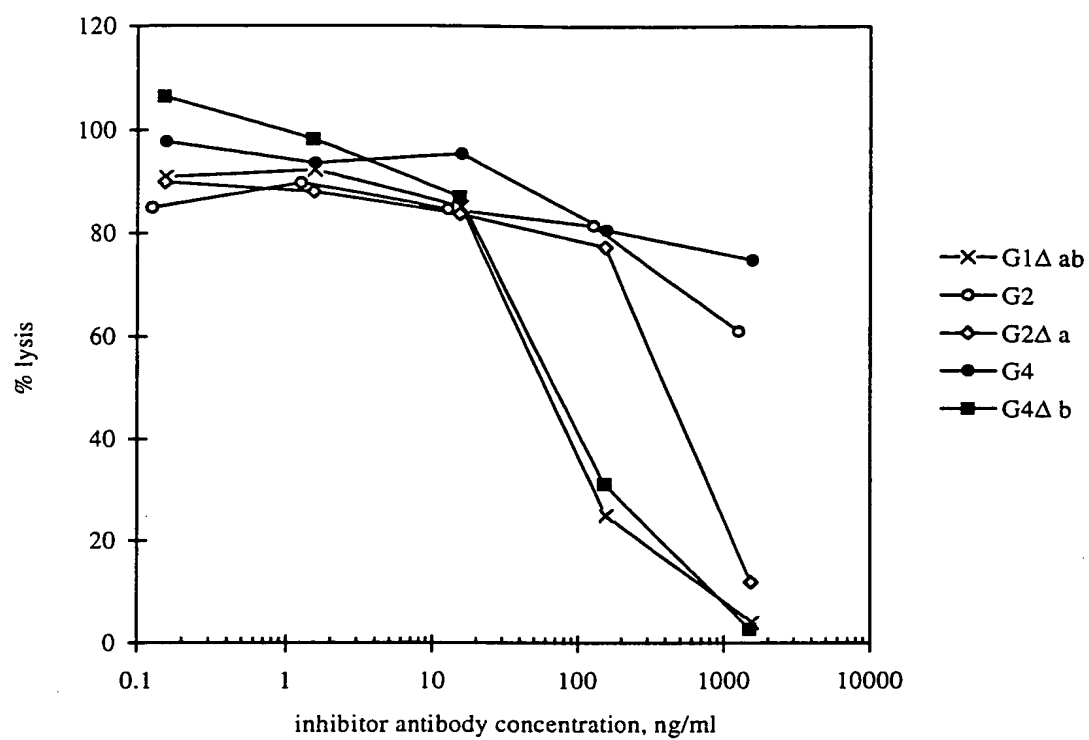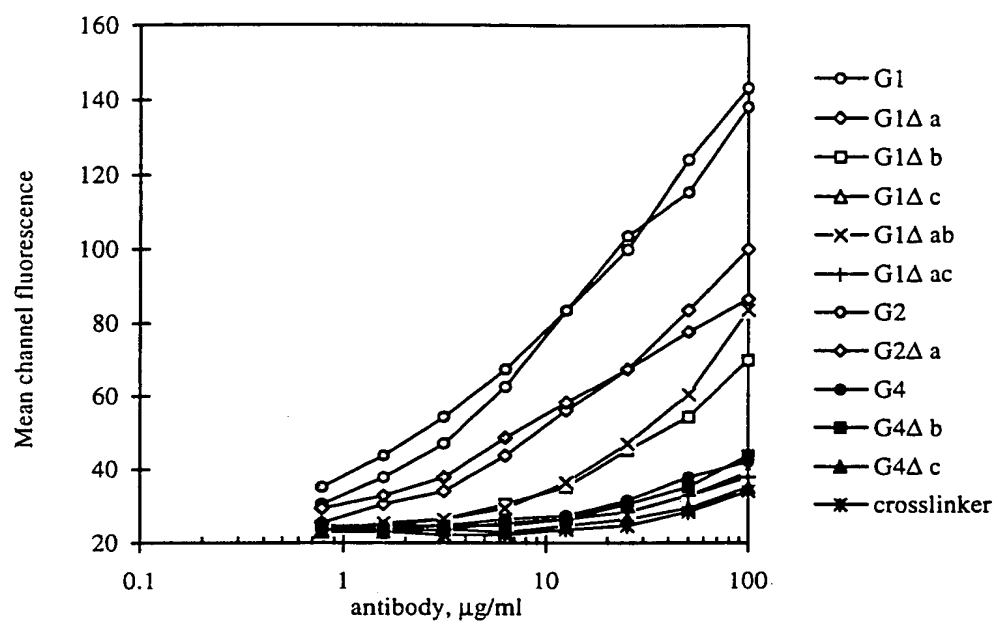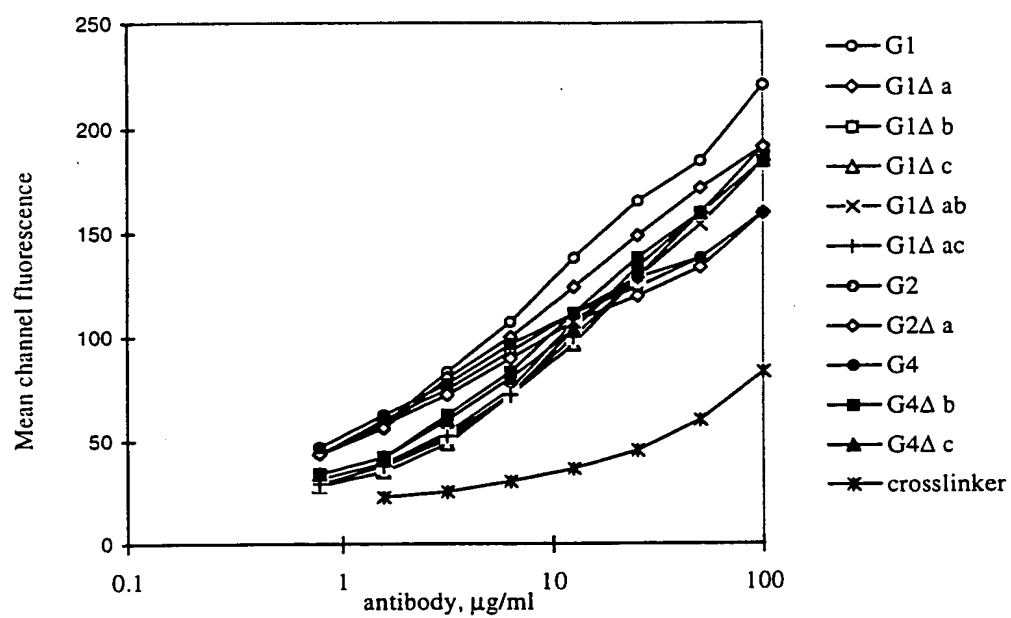
Figure 13

Figure 14

Figure 15

Table 1

A comparison of the mutations made to the wildtype G1, G2 and G4 antibodies

Antibody	233	234	235	236	327	330	331
G1	E	L	L	G	A	A	P
G1Δa	E	L	L	G	G	S	S
G1Δb	P	V	A	-	A	A	P
G1Δc	P	V	A	G	A	A	P
G1Δab	P	V	A	-	G	S	S
G1Δac	P	V	A	G	G	S	S
G2	P	V	A	-	G	A	P
G2Δa	P	V	A	-	G	S	S
G4	E	F	L	G	G	S	S
G4Δb	P	V	A	-	G	S	S
G4Δc	P	V	A	G	G	S	S

15/17

Figure 16

Table 2

Summary of antibody activities

Assay system	Series	G1	G1Δa	G1Δb	G1Δc	G1Δab	G1Δac	G2	G2Δa	G4	G4Δb	G4Δc
FcγRI: rosetting	F	+++	+++	-	-	-	-/+	-	-	++	-	-
FcγRI: fluorescent staining	C/F	+++	+++	-	-/+	-	-/+	-	-	++	-	-/+
FcγRIIa H/H: fluorescent staining	C/F	+++	++	+	-/+	+	-/+	+++	++	-/+	-/+	-
FcγRIIb1*: fluorescent staining	F	++	++	++	++	++	++	++	++	++	++	++
FcγRI/II: chemiluminescence	F	++	++	-	+/-	-	+/-	-	-	+	-	+/-
Complement lysis	C	+++	-/+	-/+	-/+	-/+	-/+	++	-	-	-	-
ADCC	C	+++	++	+/-	+	-	-/+	+	+/-	+	-	-/+
ADCC	F	++++				-/+		+	-/+	+	-/+	

Inhibition of G1 activity in assay	Series	G1	G1Δa	G1Δb	G1Δc	G1Δab	G1Δac	G2	G2Δa	G4	G4Δb	G4Δc
FcγRI: rosetting	F							-	+	-	+	
FcγRI/II: chemiluminescence	F			+++	++	+++	+++	+	+++	-	+++	+++
Complement lysis	C							-	+			
ADCC	F							-	+		++	

Series — CAMPATH-1 (C) or Fog-1 (F) antibodies tested
++++,+++, ++ or + — relative level of activity in assay
+/- — low level of activity which is significantly above background
-/+ — low level of activity which is slightly above background
- — no activity above background
blank — not tested

Figure 17

Full C$_H$2 Sequences Of the Parental and Mutated Antibodies

Position markers:
233 234 235 236   252 253 254   296 297   310 311   318 320 322   327 330 331

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G1      APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKTK
G2      APPVA_GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPRESQPNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTK
G3      APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPIEKTISKTK
G4      APEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK

G1Δa    APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPIEKTISKAK
G1Δb    APPVA_GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPIEKTISKAK
G1Δc    APPVAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPLPIEKTISKAK
G1Δab   APPVA_GFSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
G1Δac   APPVAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKOLPSSIEKTISKAK

G2Δa    APPVA_GPSVTLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVVHQDWLNGKEYKCKVSNKGLPSSIEKTISKTK

G4Δb    APPVA_GPSVTLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
G4Δc    APPVAGGPSVTLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
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81/82

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